IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: McDonald *et al.*. Group Art Unit: 1647

Serial No.: 09/360,242 Examiner: Landsman, R.

Filed: July 22, 1999

For: *METHODS AND COMPOSITIONS FOR TREATING SECONDARY TISSUE DAMAGE AND OTHER INFLAMMATORY CONDITIONS AND DISORDERS*

DECLARATION PURSUANT TO 37 C.F.R. §1.132

Commissioner for Patents
U.S. Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, JOHN R. McDONALD, declare as follows:

1. I am an inventor of and am familiar with the subject matter of the above-captioned application.

2. I received B.Sc. and Ph.D. degrees at Napier College, Edinburgh, completed successful postdoctoral appointments in Canada and The United States before leaving academia for the biotechnology industry (Boulder CO, and San Diego CA). I have been involved in all aspects of the Research and Development process from project planning through IND filing. My research has focused upon growth factor signal transduction, multiple sclerosis, and the purification and characterization of neurotrophic factors and growth factor-mitotoxin fusion proteins. I have received several peer-reviewed awards and grants, including a US National Institutes of Health Small Business Innovation Research Grant. I am co-author of over fifty publications, and a named inventor on nine patent applications.

3. I am a founder of Osprey Pharmaceuticals Limited, Canada, and I was Vice-President Research & Development and a Director at the company.

4. I have read the Office Action, mailed February 19, 2003, that issued in connection with the above-captioned application. It is my understanding that the claims are rejected by the Examiner over Roby *et al.* (Oncology Reports 3:175-179, 1996) as being anticipated or obvious. I understand that anticipation requires that a reference disclose all elements as claimed and obviousness requires a suggestion in a reference to do that which applicant has done.

5. As described below, Roby *et al.* does not disclose chemokine targeting agent-toxin conjugates that bind to chemokine receptors. The data and results presented in Roby *et al.* are inconsistent with a conclusion that its conjugates bind to chemokine receptors on cells and that its conjugates are internalized upon binding to chemokine receptors on cells. Furthermore, as described below, the C-terminus of chemokines, such as MSGA/GROα, is not responsible for receptor binding.

Roby *et al.* describes a study that evaluated the feasibility of targeted delivery of daunorubicin to melanoma cancers cells. A **C-terminal peptide** from the CXC chemokine MSGA/GROa was conjugated to daunorubicin. As demonstrated below and in the attached references, the C-terminal portion of this chemokine does not mediate binding to chemokine receptors; the data in Roby *et al.* are consistent with this conclusion. In addition, the data in Roby *et al.* do not support a conclusion that the resulting conjugate is internalized by binding to receptors on melanoma cells.

6. **Analysis**

A. Expression of Chemokine Receptors on Target Cells

IL-8, which has significant homology to MSGA/GROα, binds to the same receptors (see, Moser *et al.* (1991) *J. Biol. Chem. 266*:10666-10671 Clark-Lewis *et al.* (1994) *J. Biol. Chem. 269*:16075-16081; Kim *et al.* (1994) *J.*

Biol. Chem. 269:132909-132915). as MSGA/GRO*α*. Also, it is known that the chemokine MSGA/GROa binds to IL-8RB/CXCR2 and IL-8RA/CXCR1 with high and low affinity, respectively (see, *e.g.*, Kim *et al.* (1994) *J. Biol. Chem. 269*:132909-132915). It also is known that many chemokine receptors including CXCR1 and CXCR2 are expressed on a wide variety of cancers including ovarian cancers. In fact, both receptors have been shown to be highly expressed on human SKOV-3 ovarian cancer cells (Venkatakrishnan *et al.* (2000) *J. Biol. Chem. 275*: 6868-6875). Hence, if the conjugate of Roby *et al.* binds to chemokine receptors for MSGA/GRO*α*, then it should bind to SKOV-3 cells.

Roby *et al.*, however, shows that SKOV-3 ovarian cancer cells **are not** susceptible to the C-terminal peptide targeted drug (Tables I and II). Thus, the C-terminal peptide of MGSA/GRO*α* does not bind to chemokine receptors.

This result indicates that the C-terminal peptide that is used to target the drug in Roby *et al.* is not binding to an MSGA/GRO*α* receptor, since it would also bind to SKOV-3 cells, which express MSGA/Gro*α* receptors. Further, as discussed below, it is unlikely that the conjugate engages another protein/growth factor receptor and undergoes internalization in view of the cytotoxic kinetic data presented by Roby *et al.*

B. Chemokine receptor binding and activation sites reside on the N-terminus and NOT the C-terminus of the molecule.

As described in the application and known to those of ordinary skill in the art the chemokine superfamily is divided into four subgroups (CXC, CC, C and CX3C) based on the position of up to four conserved cysteine residues. Most chemokines belong to the CXC and CC subgroups. The different chemokines have between 15 and 50% identity in their primary structures, but they share conserved three-dimensional structures. It is their highly conserved and shared three dimensional structures that are responsible for receptor binding and function. Early structural studies were carried out with the CXC ligand, IL-8, which as noted above, has significant homology to MSGA/GROa and binds to

the same receptors (see, Moser *et al.* (1991) *J. Biol. Chem. 266*:10666-10671 Clark-Lewis *et al.* (1994) *J. Biol. Chem. 269*:16075-16081; Kim *et al.* (1994) *J. Biol. Chem. 269*:132909-132915).

Subsequent studies on several chemokines confirmed the conserved nature of the three dimensional structure among chemokines and that subtle differences in the protein conformation accounts for their functional differences. Structurally, chemokines including MSGA/GROa, are made up a flexible N-terminus (up to 11 amino acids preceding the first cysteine) followed by a conformational rigid N-terminal loop region, then 3 anti-parallel beta strands and finally, a C-terminal alpha helix. Structural studies have established that the **N-terminal region of all chemokines is essential for chemokine receptor binding, activation and internalization.** The rigid loop region following the second N-terminal cysteine of CXC and CC chemokines is responsible for initial ligand interaction or "docking" with the extracellular domain of the receptor which facilitates the access of the flexible N-terminal region (prior to the first cysteine) for receptor activation. The 30s loop (a few amino acids numbered around 30-36 from the N-terminus) is not directly involved in receptor binding, but along with the disulfides provide a scaffold that determine the conformation of the sites that are critical for receptor binding and activation. (see, *e.g.*, Clark-Lewis *et al.* (1995) *J. Leukoc. Biol. 53*:703-711; Baysal *et al.* (2001) *Proteins 43*:150-160).

The C-terminal alpha helix does NOT bind to chemokine receptors, but has a stabilizing effect on the three dimensional structure. (see, *e.g.*, Clark-Lewis *et al.* (1994) *J. Biol. Chem. 269*:16075-16081; Clark-Lewis *et al.* (1991) *J. Biol. Chem. 266*:23128-34; Zhang *et al.* (1991) *J. Biol.Chem. 269*:15918-15924). For example, functional studies on peptides of IL-8 (an analog of MSGA/GROa; as noted IL-8 and MSGA/GROa selectively bind to the

same receptors (IL8A/CXCR1 and IL-8B/CXCR2) show that a 1-51 amino acid peptide with the entire C-terminal *a* helix missing competes with the full length IL-8 for binding to the receptor and exhibits activity (see, Clark-Lewis *et al.* (1991) *J. Biol. Chem. 266*:23128-23134, 23131). Further, a 22 amino acid C-terminal peptide containing the whole C-terminal a helix **does not** to bind to the receptor or show any activity (page 23132 of the same reference). The Roby *et al.* peptide (MGSA/GRO*a* 47-71) has striking primary and secondary structure homology to the Clark-Lewis peptide.

Despite extensive structural studies on numerous chemokines over the last 15 years there have been no reports preceding or subsequent to that of the cited Roby *et al.* describing C-terminals of chemokines (or peptides thereof) that bind to any signal transducing receptor (chemokine or not). Therefore, the peptide used in the conjugate of Roby *et al.* does not bind to a chemokine receptor. Thus, the conjugate does not target chemokine receptors and is not composed of a chemokine-receptor targeting agent.

C. Kinetics of Chemokine receptor Internalization

High affinity binding of many chemokines including MSGA/GROa, results in rapid (within minutes) receptor desensitization and internalization by receptor mediated endocytosis (see, Chuntharapai *et al.* (1995) *J. Immunol. 155*:2587-2594; Mueller *et al.* (1997) *J. Biol. Chem. 272*:8207-8214; Yang *et al.* (1999) *J. Biol. Chem. 274*:11328-11333; Solari *et al.* (1997) *J. Biol. Chem. 272*:9617-9620). Internalization leads to a dramatic drop in the availability of cell surface receptors leaving the cells unresponsive to further ligand effects until the cells have time to recycle or express new receptors. In the case of CXCR2, the MSGA/GROa specific receptor, recovery was shown to take several hours without receptor expression levels reaching 100% of the initial expression levels (see, Chuntharapai *et al.* (1995) *J. Immunol. 155*:2587-2594). In the same study, MSGA/GROa was shown to be internalized and to down regulate receptors by 50% in 10 min at a concentration of 0.2 nM.

If the conjugate of Roby *et al.* was utilizing a chemokine receptor (and one of ordinary skill in the art would expect CXCR2) or any classic protein/growth factor receptor that requires internalization, the drug would have been taken up rapidly. Given the multi-mechanisms of action of daunorubicin (which is taken into cells by diffusion as outlined below) and the short term experiments described by Roby *et al.* (page 178, Table II), evidence of cell death of most of the susceptible (most metabolically active) cancer cells at lower doses would have been expected. The ID_{50} of the free and conjugated drug is in the micro-molar range and by definition so would the C-peptide (as it is conjugated 1:1 to the drug). This, however, is not what was observed in Roby *et al.* The >1000 ng/ml of ID 50 value for conjugate early incubation time points for the "target cells" in Table II (page 179) is calculated to give a concentration of at least 2000 times greater than the nM value reported by Chuntharapi *et al.* It would be charitable to expect an "active" peptide to lose this much activity when compared to the parent molecule. Roby *et al.* sum up the results of the shorter term drug incubation experiments summarized in Table II by stating "We can see from these tests that the observed tendency for long exposure (of daunorubicin and conjugate) is maintained" (page 178). This would suggest that both unconjugated and conjugated drug are taken up by the cells by a mechanism other than by a classic protein receptor route. It is most likely that the conjugated drug is taken up by diffusion as is the free drug. The Roby *et al.* data supports this conclusion.

D. Cellular uptake and Mechanism of Action of Daunorubicin

Daunorubicin (Cerubidin®, DaunoXome) is one of the lipophilic anthracycline antibiotics long used in cancer therapy (see, *e.g.*, Weiss (1992) *Semin Oncol 19*:670-686). The mechanisms of action(s) of these antibiotics include the inhibition of function and breakage of DNA, inhibition of transcription by inhibition of topoisomerase II, metal ion chelation, and generation of free

radicals (see, *e.g.*, Bakker *et al.* (1995) *Current Pharmaceutical Design 1*:1133-1144). The anthracyclines are used in cancer therapy and are toxic to a wide range of cancer cells; there are only a few unresponsive cancers (see, Weiss *et al.* (1992) *Semin Oncol 19*:670-86). Due to their lipophilic nature, these compounds are readily taken up by cells by simple diffusion (see, Bakker *et al.* (1995) *Current Pharmaceutical Design 1*:1133-1144). In fact, to exploit this property, derivatives of daunorubicin, which are on the market, have been designed to increase their lipophilic nature (see, *e.g.*, Michieli *et al.* (1996) *Haematologica 81*:295-301). The process of diffusion is time-, temperature-, concentration-dependent and energy independent (see, *e.g.*, Nagasawa *et al.* (1996) *Biol. Pharm. Bull. 19*:100-105). Efflux of the drugs is facilitated by energy-dependent membrane pumps. The intracellular concentration is determined by a balance of these two processes and is a major determinant for the cytotoxic effects or the drugs. Tumor cells show differences in their intrinsic or acquired resistance to the drug.

The data presented by Roby *et al.* show properties and functions characteristic of daunorubicin. Figures 3 through 6 and Tables I and II show that the free drug and conjugate at high concentrations are capable of killing ovarian carcinoma cells and fibrosarcoma cells as well as melanomas as would be expected of the broad spectrum cancer therapeutic. The high concentrations of drug needed in the short term experiments and the need for long term exposure is far more consistent with cellular uptake of the drug by diffusion rather than by internalization. The differences in ID_{50} values among the cell lines used could be a reflection of differences in the activity, in the accumulated intracellular drug concentration or the active state of the cells (e.g., different rates of proliferation). The result also can be a reflection of differences in the tumor

cells' intrinsic level of resistance to the drug. It must be pointed out that even resistant strains succumb to the drug albeit at higher doses (see, *e.g.*, Michieli *et al.* (1996) *Haematologica 81*:295-301; Michieli *et al.* (1999) *Haematologica 84*:1151-1158).

Roby *et al.* states that "the conjugate greatly increases the activity of daunorubicin on melanoma cells" and concludes "that the reason for such a dramatic increase is unknown" (bottom of page 178). The inference that Roby *et al.* is trying to make is that the conjugate targets the drug to these cells. These conclusions, however, clearly contradict the data in the paper. As noted, SKOV-3 cancer cells are known to robustly express the MSGA/GROa receptors, but the activity of the conjugates on these cells is decreased.

Thus, the paper is describing phenomenology. The only explanation for the data is that the conjugate is sequestered in some way. Chemokines bind to glycosaminoglycans (GAGS) including heparin and heparan sulfate, chondroitin sulfate and dermatan sulfate (see, *e.g.*, Proudfoot *et al.* (2001) *J. Biol. Chem. 276*:10620-10626). They bind with much lower affinities than to chemokine receptors. These GAGS are expressed to a varying degree on the surface of cells and are not internalized. These GAGS are thought to help localize the chemokines to the area of inflammation and disease, increase their local concentration in vivo and facilitate chemokine receptor binding. It has been shown that the binding site for these proteoglycans has been located in the C-terminal alpha helix of some chemokines where a number of basic amino acid residues (lysine and arginine, also present in the peptide discussed here). The Roby *et al.* C-terminal peptide contains these amino acid residues. In a tissue culture dish without the stabilizing and high affinity binding effect of an N-terminal portion of a chemokine, this charged molecule will bind to a number of charged surfaces including the tissue culture dishes, various proteins and GAGS in the serum and cell medium as well as to the surface of most cells. Soluble GAGS have been shown to inhibit binding to GAGS on cells.

Therefore, while it is impossible to unequivocally interpret the data of Roby *et al.* (Tables I and II), a possible explanation for the increased, decreased and no change in sensitivity among the cell lines (Table I) is differences in GAG type, expression levels, affinities and number of binding sites on the different cells and this would be after binding to miscellaneous other surfaces. Finally, given that most cells express GAGS, Roby *et al.* does not demonstrate targeted delivery.

7. **Summary**

a) Roby *et al.* does not show that a chemokine targets a toxin to melanoma cancer cells specifically nor that such conjugate of a C-terminal peptide and a drug is internalized. Given the information available at the time of publication of Roby *et al.*, one of ordinary skill in the art would not design a conjugate for targeting to chemokine receptors that lacks the N-terminal regions known to be required for cell-specific targeting (i.e., receptor binding, activation and internalization). Despite extensive work on peptides from all parts numerous chemokine proteins, there are no reports of any chemokine C-terminal peptide that is internalized or biologically active before or after that of Roby *et al.*

b) Further, Roby *et al.* does not report studies of receptor internalization, cross-linking studies or receptor identification on target cells and alleged non-target cells. The kinetics of cellular uptake and activity on different cancer cell targets exhibited by the conjugate are entirely consistent with the known properties of the free drug. Thus Roby *et al.* does not disclose, teach or suggest conjugates that target toxins leukocytes, nor that different chemokine-targeting ligands confer different cellular specificities nor use of such conjugates for treatment of disorders that share a common underlying pathology.

* * *

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

JOHN R. McDONALD

Date: May 14 03

25020-601B

Current Pharmaceutical Design, 1995, *1*, 133-144

Anthracyclines - Pharmacology and Resistance, A Review

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Abstract: Anthracyclines are anti-tumor drugs with a wide spectrum of activity in human cancers. They were introduced in clinical practice in the early 1960's and have been extensively used in the adjuvant, curative and palliative setting for both solid tumors and hematologic malignancies. Their mechanisms of action have not yet been fully clarified. The best known cellular mechanisms are prevention of replication and transcription by intercalation of DNA, formation of DNA breaks possibly due to formation of toxic topoisomerase complexes, and generation of free radicals. Pharmacokinetic studies revealed that anthracyclines dissappear fast from the plasma into tissues. Cellular uptake takes place by diffusion; efflux can take place by energy-dependent membrane pumps. The intracellular concentration seems a major determinant for the cytotoxic effect. Anthracyclines are reduced to more hydrophilic glycide metabolites and aglycones. The drug is excreted via the bile and for a minor degree via the urine. The clinical value of anthracyclines is limited by intrinsic or acquired tumor cell resistance to anthracyclines. Different ways of resistance seem to play a role in multidrug resistance (MDR) such as the overexpression of the membrane efflux pump P-glycoprotein, as well as the overexpression of the multidrug resistance associated protein (MRP) or probably other efflux pumps, a decrease in the activity of the target enzyme topoisomerase II and an increase in cellular detoxifying capacity. Resistance against anthracyclines as well as early (myelosuppression, mucositis) and late toxicity (cardiotoxicity) have directed the search for analogs with higher antitumor efficacy and lower toxicity. This search delivered variants of the original daunorubicin such as epirubicin and idarubicin with a more favorable therapeutic index, and morpholino anthracyclines, drugs designed to circumvent P-glycoprotein and non P-glycoprotein MDR. These last compounds recently enrolled phase I/II clinical trials. Results of these trials are of even more interest because up to now, results of clinical studies in solid tumors with modulators (resistance modifiers) of MDR, such as amiodarone, verapamil, cyclosporin A and PSC 833 are not very encouraging. In vivo studies with e.g. PET scanning to analyse the intratumor kinetic behaviour of anthracyclines and modulators of drug resistance, are ongoing.

Introduction

In the early 1960's an isolate from fermentation broths of *Streptomyces peucetius* and *Streptomyces caeruleorubidus*, studied for its potential activity against Gram-positive bacteries, was found to have antineoplastic efficacy in animal tumors. The simultaneous discovery of the active drug by Italian and French researchers was recorded in its name daunorubicin, a combination of the French rubidomycin and Italian daunomycin [1, 2]. Anthracyclines are still largely derived from microbial synthesis (with or without genetic manipulation), although some derivates can be completely chemically synthesized [3, 4]. Soon after daunorubicin was shown to be active in hematologic and pediatric solid tumors its side effects appeared not to be limited to mucosa and bone marrow but also included the heart [5]. Early dose limiting toxicity of anthracyclines is reversible myelosuppression (granulocytopenia) and mucositis while late dose limiting toxicity is cardiotoxicity. This toxicity pattern directed the search for analogs with less toxicity and equal or higher antiproliferative activity than their parent compound. Less than 20 derivates from more than 1000 anthracyclines that have been tested in the laboratory have been entered into clinical trials [6]. Doxorubicin (the 14-hydroxy analogue of daunorubicin) and idarubicin (4-demethoxy daunorubicin) were found to exhibit an increased therapeutic index compared to daunorubicin [7, 8]. Epirubicin, the 4'epimer of doxorubicin, demonstrated an improved therapeutic index compared to doxorubicin, and for this reason it is increasingly replacing doxorubicin [9, 10]. The first-generation structure daunorubicin has kept its place in the treatment of hemarologic malignancies, and in the same field idarubicin has been released for treatment [11]. The scope of antitumor action of both doxorubicin and its 4'epimer epirubicin as single agent or in combination chemotherapy varies from hematologic malignancies to a diversity of solid tumors including small cell lung cancer, breast, gastric, and bladder cancer, sarcomas and germ cell tumors. They are the components of adjuvant, curative, as well as palliative treatments. Limitations exist in the group of naturally ranistant tumors such as non-small cell lung cancer, colon carcinoma, renal cell cancer and malignant melanoma. Because the clinical value of all anthracyclines is limited by intrinsic or acquired resistance to the drug, search has been directed towards antitumor agents able to overcome different mechanisms of resistance. This search has yielded the morpholino anthracyclines (MA) from which methoxymorpholino doxorubicin (MMD) and MX2 (3'-deamino-3'-morpholino-13-deoxo-10-hydroxycarminomycin) have recently entered phase I/II clinical trials [12-14]. Except idarubicin and possibly MX2, anthracyclines are not able to pass the blood brain barrier and thus exert no activity against intrathecal tumors [15, 16].

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Anthracycline Derivatives

Anthracyclines have in common a hydrophobic part, the fluorescing tetracyclic chromophore (rings A,B,C and D) which gives the drug its characteristic bright color [17](Fig. (1)). Linked via a glycosidic bond is the hydrophilic aminosugar daunosamine. Daunorubicin and doxorubicin only differ in the presence of one hydroxyl group at the C-14 position of the A ring [18]. Differences in structure have consequences for the cytotoxicity of the drugs in a direct way or indirectly by changing its chemical or pharmacologicalcharacteristics. Knowledge about structure-activity relationships has determined the direction of the search for derivatives with more favorable profiles. It is thought that changes at the 4'-position of the daunosamine sugar influences the affinity of the drug for DNA. An example is the second-generation anthracycline epirubicin that possesses an equatorial instead of axial configuration of the hydroxyl group of the aminosugar [19]. Idarubicin differs from

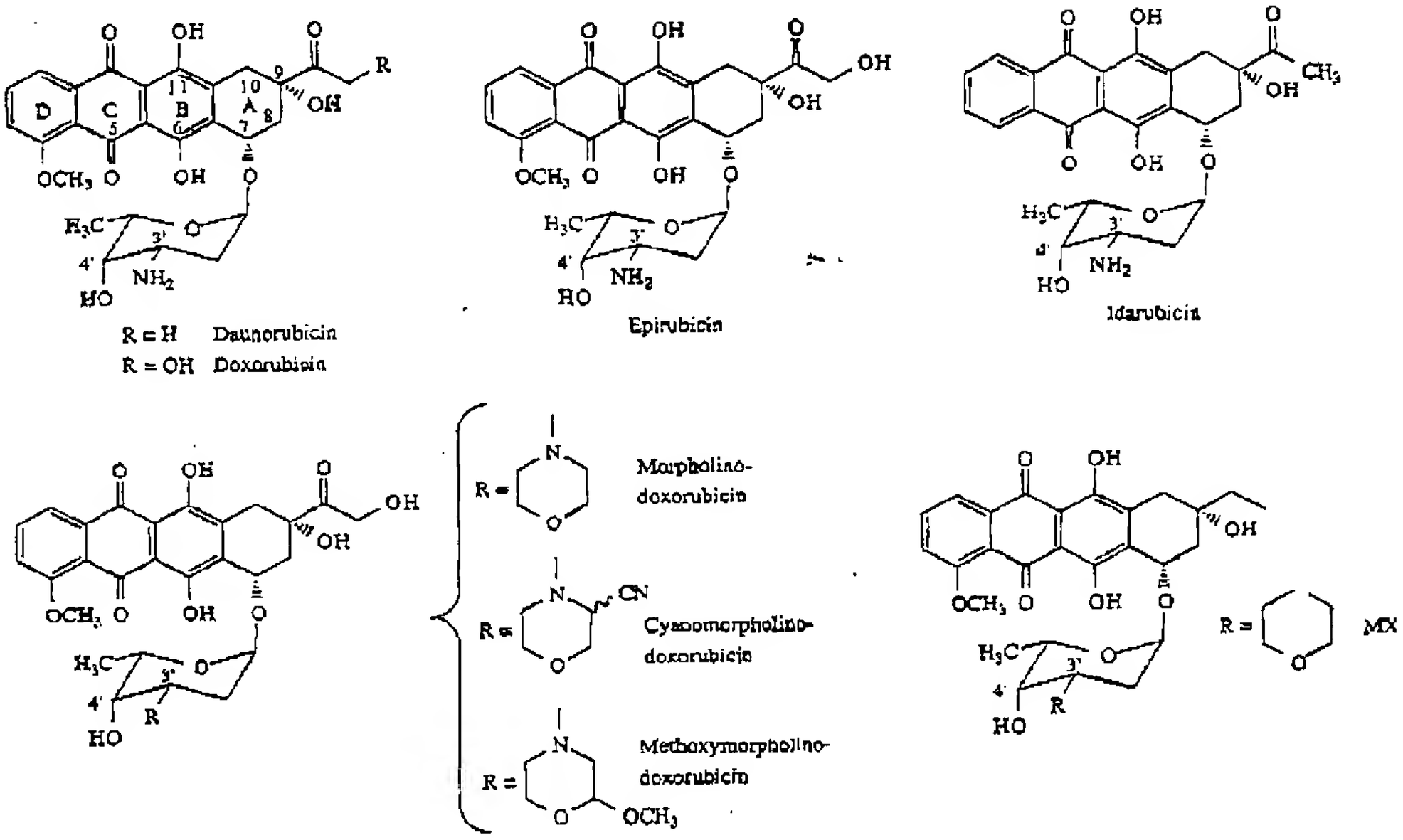

Fig. (1).

daunorubicin in the substitution of a hydrogen for the methoxy (-OCH_3) group of the D ring of the chromophore. This change improves the acid stability and increases the lipophilicity of the drug which enables oral administration [8]. The MA possess a morpholinyl ring substituted at the 3'position of the daunosamine sugar; like idarubicin (and unlike other anthracyclines) these analogs retain their potency when orally administered [20].

Mechanisms of Action

Table 1 summarizes the different mechanisms by which anthracyclines exert their cytotoxic action. A number of actions that constitute the cytotoxic properties of the drug are described; however, it is not clearly distinguishable which of these actions is the most important in inducing cell damage. The main target for cytotoxic action of the anthracyclines is DNA. Irreversible DNA damage more than a reduction in the amount of synthesized DNA however seems to be the basis of the cytotoxicity [21]. The best known mechanism is the intercalation between two base pairs of DNA or ribonucleic acid (RNA). The rings of the polycyclic chromophore play a vital role in the intercalation by binding to DNA and RNA. By a strong electrostatic bond of the positively charged amino sugar portion of the anthracyclines to the sugar-phosphate backbone of DNA the intercalated molecule is stabilized at intracellular pH. This way vital actions such as replication and transcription are blocked [22, 23]. Recently, Skladanowski demonstrated DNA crosslinking of anthracyclines using a mild DNA denaturation method that kept temperature and pH unstable bonds intact. Before, no crosslinking could be demonstrated due to more aggressive methods of DNA denaturation [24].

Table 1. Mechanism of Action of Anthracyclines

Mechanism	Effect
Intercalation between base pairs of DNA and RNA	Inhibition of DNA replication
DNA interstrand crosslinking	Inhibition of DNA replication
Cleavable complex formation with topoisomerase I/II	Single and double strand DNA breaks
Free radical formation	Destruction of macromolecules, DNA and RNA breaks
Enzyme inhibition	Inhibition of DNA/RNA synthesis, inhibition of (synthesis of) detoxifying enzymes
Binding to membranes	Alteration of membrane function
DNA alkylation	Destruction of DNA

Topoisomerases are essential for DNA metabolism by inserting transient breaks into single (topoisomerase I) or double strand (topoisomerase II) DNA, thus solving mechanical problems caused by the double-helical structure of DNA [25]. The anthracycline-topoisomerase complexes (cleavable complexes) form stabilized DNA breaks, thus turning topoisomerases into cellular toxins. The nature of the substituent at the 3'-position of the daunosamine moiety and of the C-14 position of the chromophore ring A are essential in the formation of cleavable complexes [26, 27]. All anthracyclines but MAs seem to exert

their cytotoxic action partly by inhibiting topoisomerase II by the formation of cleavable complexes.

Anthracyclines also exert cytotoxicity by radical formation through electron transport with a vital role for the quinone (C) ring [28]. The quinone ring can be reduced by nicotinamide-adenine dinucleotide phosphate (NADPH)-dependent flavin reductase to a semiquinone ring by taking up a single electron whereafter this semiquinone ring can be further reduced by taking up a second electron, or donate its free electron to molecular oxygen thus generating a superoxide radical [29, 30] (Fig. (2)). In the absence of oxygen, anthracyclines have the potency to generate aglycone free radicals (by splitting off the amino sugar of the semiquinone radical) that produce DNA and RNA single and double strand breaks. Free oxygen radicals can damage intra- as well as extracellular macromolecules (lipids and proteins). By their action cells and mitochondria swell, other organelles fragmentate, and essential intracellular enzymes are inhibited [31]. Among these enzymes are DNA and RNA polymerases, repair and detoxifying enzymes and nuclear matrix related enzymes such as topoisomerase I and II. Anthracyclines can also be cytotoxic by binding to membranes thus altering their functions. Tritton et al. demonstrated cytotoxicity by membrane interaction in vitro where entrance of free doxorubicin was prevented by coupling doxorubicin to an insoluble agarose support [32].

life of epirubicin is shorter than of doxorubicin (epirubicin: 30-40 hours, doxorubicin: 40-70 hours), with an identical volume of distribution [40]. At physiological pH a lower fraction of epirubicin is in its ionized form compared to doxorubicin which makes the drug more lipophilic and facilitates cellular uptake [9]. In accordance with these data are higher plasma levels for doxorubicin and higher plasma clearance and elimination rates for epirubicin compared to doxorubicin. Elimination is mainly through the bile; a small amount is excreted by the kidneys (5-11 %) [41, 42]. The excretion of iv administered doxorubicin in the bile was determined to be 41 % in 7 days, and in the urine 13.7 % in 7 days [43]. Doxorubicin and its active alcohol metabolite are excreted for a small part into the saliva, thus exposing locally the mucosa of the upper gastrointestinal tract to the drugs for at least 2 days after iv delivery [44]. Liver function disorders can seriously delay elimination of the drug and its metabolites, necessitating a dose reduction. Epirubicin clearance in patients with hepatocellular carcinoma correlated best with serum aspartate aminotransferase (AST) levels, suggesting AST serum levels could possibly be a better basis for dose reductions than the currently used serum bilirubin levels [45]. In case of renal insufficiency, however, full dosages can be given [46]. Inter-individual variability of plasma pharmacokinetics is high, whereas intra-individual variability is much less pronounced. Inter-individual variation could be reduced if sex and age are included in a clearance model [47].

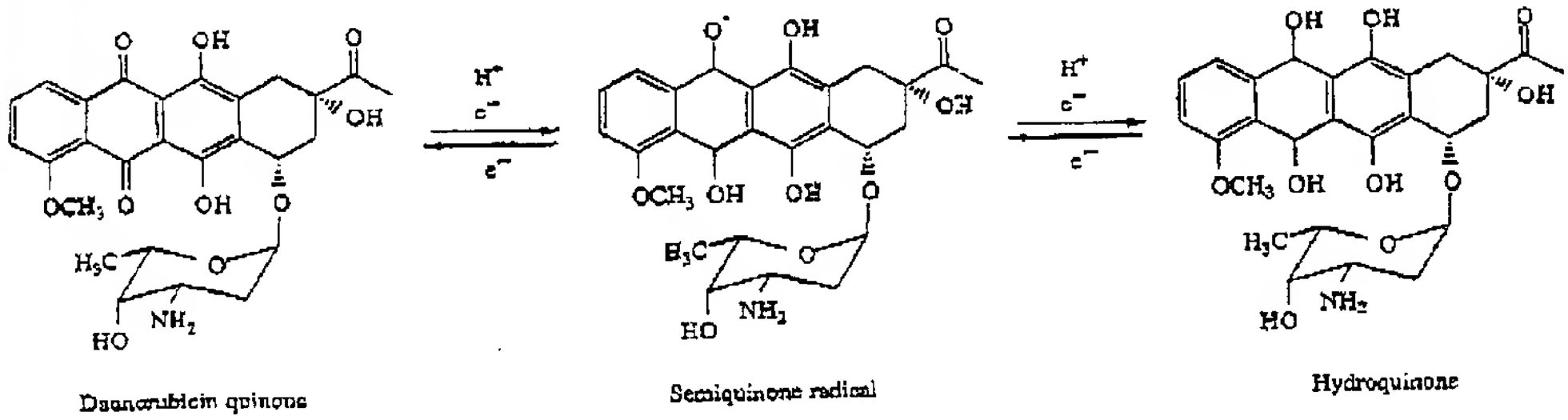

Fig. (2).

The mechanisms of action of the MA are in some ways different from those of other anthracyclines. The morpholinyl ring substituted at the 3' position of the daunosamin unit of the MAs precludes their interaction with topoisomerase II [33]. Inhibition of topoisomerase I plays a role for these derivates; it remains unclear whether topoisomerase I inhibition plays a role in cytotoxicity for doxorubicin as well [34]. MAs intercalate into DNA comparable with other anthracyclines and cause single-strand breaks; after microsomal activation they can alkylate and cause DNA interstrand cross-links. The cyanomorpholinyl anthracyclines however can bind covalently to single strand DNA followed by the formation of DNA-DNA interstrand cross links without preceding metabolic activation [35, 36]. Ribosomal gene transcription is selectively inhibited by MA, a process that is observed in vitro within 30 min drug exposure [37].

Plasma Pharmacokinetics

Anthracycline concentrations are usually determined by high-performance liquid chromatography and fluorescence detection (using the natural fluorescence of the anthracyclines) [38]. After intravenous (iv) administration anthracyclines disappear in a triphasic serum decay pattern. They move very fast from the plasma into tissues with an initial half life of 3-5 min for both doxorubicin and epirubicin [39]. The terminal half

Because of the considerable variation individualization of drug administration using individual patients' pharmacokinetic parameters was suggested. A limited sampling model for epirubicin and doxorubicin pharmacokinetics, using only two samples to estimate the area under the curve (AUC) of the drug has been proposed [48-50]. Twelves et al. found dose-dependent or non-linear pharmacokinetics with higher clearance for higher dosages when plasma levels after two bolus injections were measured. Other studies however report dose-independent or linear pharmacokinetics [43, 51]. Legha et al. reported a 10-fold decrease while Twelves et al. reported a 40-fold decrease in peak plasma levels when doxorubicin was given as a 4-day infusion compared to the usual schedule of a 3-weekly bolus of the same drug intensity (25 mg/m^2/week) [52, 53]. Eksborg [54] observed an up to 10-fold decrease in peak plasma value when doxorubicin was administered as a 4-hour infusion; only minor effects on peak plasma levels were obtained when the infusion duration was further prolonged. Cytotoxic action is thought to be determined by total drug exposure estimated by the AUC but the relation between pharmacokinetic parameters and cytotoxicity is not clear. In vitro studies have shown a therapeutic advantage for long-term low-dose treatment compared to short-term high-dose treatment [55, 56]. Little however is known about the effect of modulation of infusion schedules on response rates and survival in vivo [57]. While some studies report a correlation between AUC, plasma clearance, terminal rate constants and elimination constant on one side and response status on the other side [58,

59], other studies have failed to demonstrate a correlation between plasma pharmacokinetic parameters and response rates. Moreover, tissue to plasma ratios differ for different organs. This further reduces the value of a mere estimation of the AUC. Reports on the relationship between pharmacokinetic parameters and toxicity are no more univocal. Cardiac toxicity was found to be partially determined by peak plasma levels. Low-dose long-term infusion is related to an increased AUC and lowered plasma peak levels compared to the pharmacokinetic parameters of a bolus injection of the drug. Therefore, clinical studies have been performed with such schedules to reduce cardiac toxicity with maintenance of or increase in cytotoxic activity [60-62]. Continuous infusion has advantages; it is devoid of the discomfort of frequent venapunctures and the use of portable pumps and central venous catheters made treatment on an outpatient basis possible. Infusion by long-term venous access catheter, however, has its own side effects such as subclavian vein thrombosis, although the incidence seems to be low [63].

Studies have been performed to test the benefit on tumor cell uptake and toxicity of packaging or targetting anthracyclines. Encapsulating doxorubicin in liposomes, small vesicles composed of phospholipids, could possibly result in lower cardiotoxicity and higher tumor cell uptake. The pharmacokinetic profile of liposome encapsulated doxorubicin (LED) differs from the pharmacokinetics of the free compound. Peak plasma levels and AUC values of LED are found to be up to 20 times higher than levels found after infusion of free doxorubicin [64]. The volume of distribution at steady state is markedly decreased for LED compared to doxorubicin, with retarded conversion to its alcohol and aglycone metabolites, thus effecting prolonged exposure to the far more toxic unmetabolized doxorubicin. Depending on size and composition of the liposomes, uptake will take place preferentially in the liver and spleen (the reticuloendothelial system, (RES)), and in certain tumors. Increased uptake of the liposomes in organs other than the RES can be achieved by changing the size and composition of the liposomes to enhance a prolonged circulation time [65]. In mice a selective uptake in tumor areas with improved therapeutic index for LED was observed [66]. In a phase I study in patients with high tumor involvement of the liver a higher maximum tolerated dose (MTD) (120 mg/m^2) was found for LED than for doxorubicin (60-90 mg/m^2) [67].

In vitro studies and in vivo studies in nude mice bearing human tumors showed enhanced activity of anthracyclines conjugated to antibodies against tumor cells compared to the free drug [68, 69]. Oldham et al. demonstrated the feasibility of delivering individually specified drug immunoconjugates in patients with refractory disease [70]. Unpredictable hematologic toxicity by variable dissociation of doxorubicin from the antibody appeared to be the limiting factor. Other carriers such as autologous erythrocytes loaded with doxorubicin have been used to produce special organ targetting [71]. Research in this area is still ongoing.

Cellular Pharmacokinetics

The transport mechanism by which anthracyclines enter the cell is not fully revealed. Cellular uptake seems to take place by Fickian diffusion or by carrier-mediated passive transport. Some investigators report saturation kinetics and substrate competition, and therefore adopt a carrier-mediated transport system model. The uptake is dependent on temperature, pH and cell cycle phase [72, 73]. Uptake (and lethality) is maximal in cells in S-phase; uptake is higher at higher temperature and in a slightly more alkalic environment when anthracyclines are electroneutral and hydrophobic. Cells are able to build high cellular to extracellular ratios of drug concentrations, suggesting active transport mechanisms. However, active transport mechanisms into the cell have never been demonstrated, and high intra- to extracellular ratios will probably be built up due to the strong binding of anthracyclines to intracellular structures. Doxorubicin has a slightly higher dissociation constant than daunorubicin and epirubicin; therefore its intracellular retention is prolonged, which might explain its favorable effect on slowly growing solid tumors. A facilitated cellular uptake of epirubicin compared to doxorubicin can explain an increase in cytotoxicity observed when a tumor cell line was incubated for one hour with epirubicin compared to doxorubicin, while toxicity was found to be equal for both drugs when the incubation period was extended to 24 hours [74]. Higher intracellular peak concentrations for epirubicin compared to doxorubicin were observed in patients with acute leukemia [75]. High lipophilicity of the MAs facilitates rapid diffusion through the cell membrane, where they reach high intracellular levels. The lipophilicity and the cellular accumulation of the drug do correlate. Some authors report a correlation between cellular accumulation and cytotoxicity [76, 77]. Within the cell, most of the drug is found in the nucleus, but considerable amounts have been found in the cytoplasm, the plasma membrane, mitochondria and other organelles [78, 79]. A direct correlation between the amount of DNA-bound anthracyclines and cell death is reported [80]. It is thought that an ionized aminogroup in the anthracycline molecule is an important factor for binding to the DNA.

Metabolism

Anthracyclines are extensively metabolized. Fig. (3) summarizes the major metabolic pathways of the anthracyclines. Metabolization of the different anthracyclines is comparable and primarily takes place in the liver. The interindividual variation of pharmacokinetic data of metabolites is as large as the interindividual variation of unmetabolized anthracyclines. Anthracyclines are metabolized by three types of metabolic reactions: keto reduction, hydrolytic or reductive cleavage, and conjugation [81]. A reduction of the C-13 ketone group to a hydroxyl group by the enzyme aldoketoreductase delivers the 13-dihydro (alcohol) metabolites. For daunorubicin, doxorubicin and idarubicin the major metabolite found in plasma is the alcohol metabolite. While daunorubicinol is found at higher concentrations in plasma than the unmetabolized drug, doxorubicinol concentrations are found to be well below one-third of the concentrations of the parent drug [82]. For idarubicin, equal concentrations are found of the parent drug and the alcohol metabolite. The terminal half lives of the alcohol metabolites of doxorubicin and epirubicin were found to be up to 1.5 times longer compared to the unmetabolized drugs [41]. Both the unmetabolized anthracycline and its alcohol metabolite are substrates for reductive and hydrolytic cleavage catalyzed by NADPH. The products are the 7-deoxy metabolites or 7-deoxy aglycones of the anthracyclines and their alcohol metabolites and the 7-hydroxy aglycones. Further on, reductive cleavage of the 4'-position can result in 4-7 deoxy aglycones or 4-deoxy, 7-hydroxy aglycons. Mross et al. [41] found a second peak in the AUC of aglycones of doxorubicin and epirubicin metabolism 2-12 hours after injection, suggesting enterohepatic circulation of these metabolites.

A minor metabolic pathway is formed by conjugation: best known is the conjugation of epirubicin with reduced glutathione (GSH) to form detoxified GSH-epirubicin adducts. Epirubicin glucuronide was found to be the major metabolite of epirubicin in plasma; concentrations of the glucuronide exceeds those of the unmetabolized drug [82]. Unlike doxorubicin and epirubicin and their metabolites which reach their peak within minutes after bolus injection of the drug, the glucuronides have their maximal

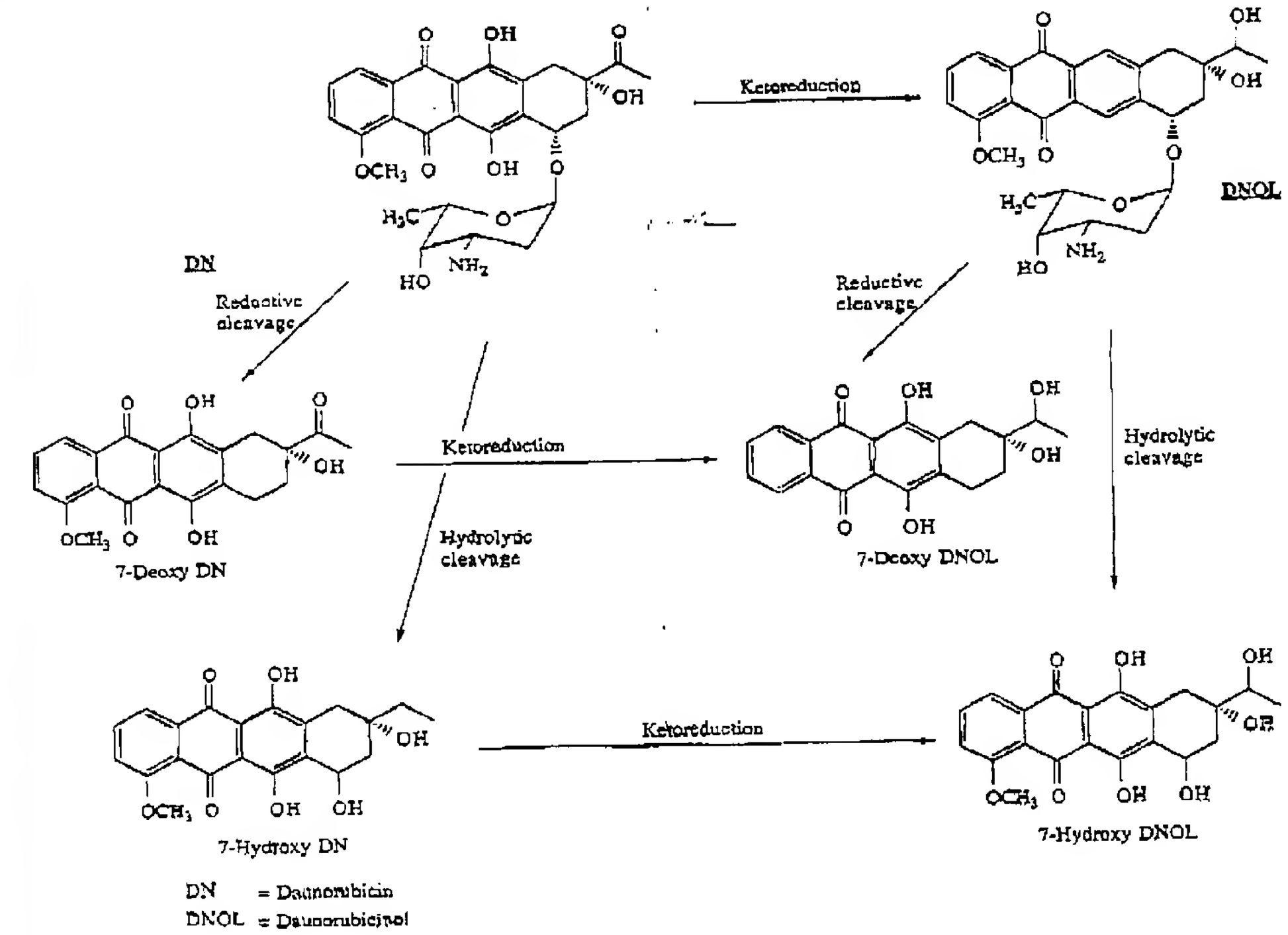

Fig. (3).

plasma concentration 1-2 hours after bolus administration. The glucuronide group was found to attach to the 4'position of the daunosamine sugar; the equatorial orientation of the hydroxyl group at this position of epirubicin enables this conjugation. Epirubicin glucuronides account for 97.3 % of the compounds measured in the first 3 hours following a 1 hour-infusion of 50 mg/m^2 epirubicin [83]. Glucuronidation might enhance the elimination rate of epirubicin which might be related to its lower toxicity profile [84]. Both the alcohol metabolite and the aglycones are more hydrophilic than the unmetabolized drug; the alcohol metabolites show lower toxicity than the unmetabolized drug while the aglycones exert hardly any cytotoxicity. Robert et al. [85] found a positive correlation between low rates of epirubicin glucuronidation and better tumor response rates. The reduction in cytotoxic potential of the alcohol metabolite and the aglycones may be partially due to their lower cellular uptake and nuclear localization [86]. The alcohol metabolites of doxorubicin (doxorubicinol) and epirubicin (epirubicinol) were found to exhibit 5 % or less of the activity of their parent drugs in tumor cells [87]. The relative toxicity of daunorubicinol compared to daunorubicin was slightly higher (up to 14 %). Only idarubicinol, the alcohol metabolite of idarubicin, was found to be as cytotoxic as unmetabolized drug. Ketoreductase conversion of idarubicin can therefore not be regarded as an inactivation pathway as is the case for other anthracyclines. Studies investigating in vitro tumor cell metabolism generally report undetectable or low levels of cellular metabolism of anthracyclines [88, 89].

Toxicity

Table 2 shows the toxicity profile of anthracyclines. The early dose-limiting toxicity is (leuko- and) granulocytopenia and mucositis. Not only the actual numbers of the granulocytes are decreased but in vitro studies also showed that their function

Table 2. Toxicity Profile of Anthracyclines

ACUTE TOXICITY
Bone marrow depression (mainly leuco- and neutropenia)
Alopecia
Nausea and vomiting
Stomatitis
Phlebitis: tissue necrosis after extravasation
CHRONIC TOXICITY
Cardiomyopathy
No secondary leukemias

is impaired by the toxic effects of anthracyclines [90]. Other side effects are nausea, vomiting, phlebitis and alopecia. Reducing the blood flow to the scalp (by using cold caps), respectively to the mucosa of the mouth (by swallowing ice-cubes) during infusion of the drug have not proven any substantial benefit [91, 92]. Extravasation of the drug often leads to tissue necrosis. Myelosuppression and mucositis as

major early dose limiting toxicities were not reduced by prolonging the time of infusion in the majority of the short versus long-term infusion studies. Some studies even report an increased risk of mucositis when time of infusion was prolonged [93, 94]. LED reduces the risk of phlebitis and necrosis from extravasation by a reduction of the sclerosing effect of doxorubicin. In intraperitoneal or intravesical use, where dose limiting toxicity is chemical peritonitis or cystitis, LED permitted higher dosages compared to free doxorubicin [95]. With iv LED a higher incidence of fever with a lower incidence of nausea and vomiting was found. Acute dose limiting toxicities were leuko- and neutropenia and stomatitis [67]. This is in contrast to results from an phase I study with iv LED in which a striking absence of stomatitis was reported [64]. Another well-known side-effect is the so-called recall phenomenon: the reactivation of radiation reaction in any previously irradiated field by anthracyclines [96]. Cardiotoxicity has been described as an acute, subacute or late (more than ten years after chemotherapy) irreversible biventricular congestive heart failure (CHF) [97]. CHF has been observed in patients with and without signs of (subclinical) cardiotoxicity during or shortly after their anthracycline treatment. Subclinical cardiac damage is usually defined as an equal to or greater than 10 % absolute decrease in left ventricular ejection fraction measured by radionuclide cineangiocardiography. The most sensitive method to predict CHF is an endomyocardial biopsy. Morphologic changes observed in chronic cardiotoxicity from anthracyclines are cardiac dilatation, degeneration and atrophy of cardiac myocytes, interstitial edema and fibrosis [99]. The potential reversibility of left ventricular dysfunction has been suggested [98], but improvement of ventricular ejection fraction seems to be a functional adaptation, more than an anatomical repair. The exact mechanism(s) by which anthracyclines exert their cardiotoxic action are not completely revealed. Free radical formation seems to play a major role [100]. NADPH dependent reductases are able to convert anthracyclines to semiquinone radicals. Mitochondria might represent the major site of semiquinone radical generation. The heart seems to be less capable of defending itself against free radicals by smaller amounts of detoxifying enzymes than the liver that is also rich in mitochondria and reductases. The risk of cardiotoxicity increases with increasing cumulative dosages and appears to be age-related with higher risks for younger children [101]. The cardiac function of 115 children who had been treated for acute lymphoblastic leukemia with doxorubicin was studied 1 to 15 years after their treatment [102]. Abnormalities of left ventricular afterload or contractility were found in 57 % of the patients with cumulative dose of doxorubicin as predictor of abnormal cardiac function. Other well-known risk factors are previous mediastinal irradiation, hypertention, electrocardiographic changes and signs of left ventricular hypertrophy at baseline. Based on a study in 135 breast cancer patients treated with epirubicin it was supposed that cumulative dosages of epirubicin over 500 mg/m^2, and especially over 1000 mg/m^2, form a risk of cardiotoxicity and of death due to CHF [103]. Von Hoff et al. had determined the cumulative maximum tolerated dose for doxorubicin at 500-550 mg/m^2 [104]. Whether cardiotoxicity could be reduced if doxorubicin was administered by prolonged versus bolus infusion was evaluated in several studies [105, 106], assuming a relationship between cardiotoxicity and peak plasma values of the drug. Pacciarini et al. found less doxorubicin taken up by the myocardial cells of mice when peak plasma levels were lowered by giving the same dose in small daily fractions compared to the uptake after a single bolus injection [107]. It is supposed that detoxifying mechanisms protecting the heart against toxic effects of anthracyclines and/or their metabolites become saturated at higher levels of the cytotoxic drugs. For daunorubicin, doxorubicin and epirubicin the relative cardiotoxicity was found to be highest for the 13-dihydro metabolite (dauno-, doxo- and epirubicinol, respectively) [108]. A direct relationship was found between the degree of cardiotoxicity and accumulated relative amounts of anthracycline metabolites [109]. Clinical trials showed that continuous infusion of doxorubicin lowered the incidence of (sub)clinical cardiac damage compared to bolus infusion. Billingham et al. described a histopathologically method that allowed quantification of cardiac damage in endomyocardial biopsies [110]. Different studies were able to show a reduction of endomyocardial damage for more frequent low-dose or continuous treatment [111, 112].

Scheduling appeared to have a major effect on cardiotoxicity with a decrease of clinical and histological cardiac damage if time of infusion was extended to 24 hours or more [48].However, no reduction of cardiotoxicity was seen when the duration of infusion was less than 24 hours. Cardiac protection of LED might be related to the retarded drug uptake by cardiac tissues due to the interaction of the drug with the cardiolipin component of the vesicles [64]. Doxorubicin form doxorubicin-iron chelates that have the capacity to bind to the surface of cell membranes where they catalyze the reduction of oxygen to superoxide radicals by GSH, with a resulting membrane destruction. The bisoxopiperazine class of drugs, especially dexrazoxane (ICRF-187), is able to distract iron from the doxorubicin-iron complex by its iron chelating potency, thus preventing the membrane destruction by doxorubicin [113]. ICRF-187 seems the most promising of the group of scavenger drugs tested for their clinical potential to reduce cardiotoxicity in anthracycline treatment. ICRF-187 was originally developed as an anticancer agent, but while it exhibited only little cytotoxic activity it appeared to reduce cardiotoxicity of doxorubicin and daunorubicin in animal studies [114]. The first clinical studies with this cardioprotector show equal noncardiac toxicity and cytotoxic efficacy compared to treatment with anthracyclines without ICRF-187 [115], while in one study ICRF-187 was found to permit administration of higher cumulative dosages of doxorubicin [116]. Testing new anthracycline analogs with unknown cardiotoxic profile demands intensive and prolonged monitoring to detect cardiotoxic effects of the drug [117]. In mice pharmacokinetic data of heart tissue (AUC of intracellular anthracycline concentration) combined with measured cardiotoxicity relative to doxorubicin was found to be a promising model to predict cardiotoxic potency of new anthracycline analogs [108].

Mechanisms of Resistance

Anthracyclines are involved in multidrug resistance (MDR) [118]. Drugs involved in MDR share no structural or functional commonality, but share their natural character (plant alkaloids, antibiotics of bacterial or fungal origin), their lipophilicity and their relatively large molecule weight. They are amphipathic, positive or uncharged at neutral pH and enter cells without the use of carriers [119, 120]. Their transport over the cell membrane appeared to be controlled by energy-dependent efflux pumps such as the P-glycoprotein (encoded by the mdr1 gene). The main characteristic of this type of resistance is a decreased cellular accumulation of drugs that are substrate of P-glycoprotein (e.g. anthracyclines, vinca-alkaloids, taxanes, epipodophyllotoxins). The best known mechanism of action of MDR is that of the P-glycoprotein efflux pump in the plasma membrane [121]. As P-glycoprotein is part of the cell membrane of normal tissues with a variety of functions (e.g. liver, kidneys, adrenal glands, blood brain barrier and, with low expression, in the bone marrow) its natural role is thought to be a bouncer for potentially toxic agents. Fojo et al. [122] demonstrated high levels of P-glycoprotein in tumors originating from tissues with

a natural high expression of the protein. Both renal and colon cancer are examples of tumors with high P-glycoprotein expression that are intrinsically resistant to chemotherapeutic agents. A positive correlation was observed between P-glycoprotein expression and refractoriness to chemotherapy in several hematologic and solid malignancies, and increasing levels of P-glycoprotein expression were demonstrated with increasing exposure to chemotherapeutic drugs [123-126]. Some studies did support the hypothesis that P-glycoprotein expression not only correlates with response to chemotherapy but also with the grade of differentiation of the tumor and with local tumor aggressiveness [127, 128]. The entire coding sequence of P-glycoprotein has been deciphered from a highly drug resistant human cell line [129]. Transfection of the mdr1 gene in sensitive cells turned them and their offspring into multidrug resistant cells [130].

Recently, overexpression of messenger RNA (mRNA) encoding for another protein involved in MDR, multidrug resistance-associated protein (MRP), was demonstrated by Cole et al. in a human lung cancer cell line [131]. In this cell line no reduced anthracycline level was observed, probably because of compartimentalization in vesicles. Recently, Grant et al. and Zaman et al. have successfully transfected cells with expression vectors containing MRP cDNA resulting in MDR cells [132, 133]. Zaman et al. observed increased drug efflux in the transfected cell line.

A second mechanism of resistance against anthracyclines has been defined by a decreased cellular concentration or altered function of topoisomerase II [134, 135]. Within one cell line both quantitative and qualitative changes in topoisomerase II can occur simultaneously [136]. The activity and the amount of the enzyme is cell cycle dependent and maximal at G_2/M phase [137]. Cells with an increased amount of topoisomerase II have been found to be hypersensitive to intercalating agents [138]. Cell lines with decreased activity of topoisomerase II have shown decreased cytotoxicity in response to treatment with these chemotherapeutic agents compared to cell lines with a normal level of topoisomerase II level [139-141].

The availibility of cellular detoxifying systems is a third mechanism of resistance against anthracyclines [142]. De Vries et al. [143] described an initially sensitive human lung cancer cell line that showed increasing GSH and sulfhydryl levels, and increasing activity of glutathione S-transferase (GST) and catalase during chemotherapeutic treatment of the patient with clinical development of complete resistance. It is suggested that increased activity of these detoxifying pathways increases the tolerance for anthracyclines [144]. The GS-X pump is a pump for removal of GSH conjugates [145]. Jedlitschky et al. and Müller et al. have recently demonstrated that overexpression of the MRP gene in a human cancer cell line increases the activity of the GS-X pump in plasma membrane vesicles isolated from these cells, suggesting a link between MRP and the GS-X pump [146, 147].

Intracellular compartimentalization may be another mechanism of resistance. An in vitro fluorescence study of daunorubicin showed uptake in cytoplasm and nucleas in sensitive cells, associated with enhanced retention and cytotoxicity, whereas resistant cell lines showed diversion from the nucleus and accumulation into membranes and the Golgi apparatus, which is associated with reduced cytotoxicity [148].

Cell lines that have shown resistance against the MAs, synthesized to retain activity in resistant tumors, should reveal the mechanisms involved in resistance to these agents. Acquired in vitro and in vivo resistance against both cyano- and methoxymorpholinyl doxorubicin (MMD) was observed in a murine cell line, L1210, that had been repeatedly treated with MMD [149]. Interestingly, resistance against morpholino doxorubicin was only present in vivo, but could be established in vitro when liver fractions were added to the cells. These cells showed no expression of P-glycoprotein, were sensitive to topoisomerase II inhibitors and had normal levels of GST and GSH. Observations concerning DNA damage and repair mechanisms left the possibility of a resistance mechanism related to an increase in DNA damage. Lewis et al. [150] and other groups observed in ES-2R, an ovarian carcinoma cell line resistant for cyanomorpholinyl doxorubicin, cross-resistance for alkylating agents and ionizing radiation. These cells had lowered topoisomerase II levels, increased GSH levels with increased GST activity and enhanced DNA repair; sensitivity could be regained by modulating GSH metabolism. Cole found resistance against cyanomorpholinyl doxorubicin in a cell line that was later found to overexpress MRP [151]. The exact mechanisms of resistance to MA still have to be identified. Nowadays, the use of the polymerase chain reaction as a highly sensitive detection method for P-glycoprotein enables the detection of very low levels of expression. The clinical relevance, however, of very low levels of expression still has to be revealed [152].

Resistance to anthracyclines in vivo will probably seldom be the result of a single resistance mechanism; different mechanisms are thought to be involved in drug resistance in human tumors [153].

Circumvention of Resistance

Circumvention of resistance is a major goal in cancer research. Increasing the dose of currently available anthracyclines has been used as a way to circumvent resistance. Epirubicin, especially in high-dose therapy (i.e.: dosages above the convential dose of 60-90 mg/m^2/3 weeks), shows a clear dose-response relationship [9]. Doxorubicin, with its relatively higher toxicity profile, can not be escalated to dosages that might show significantly better response rates. Recently, high-dose epirubicin studies in solid tumors and hematologic malignancies have been performed to evaluate this supposed increase in therapeutic index [154-156]. Higher response rates than those achieved with conventional doses of epirubicin were obtained in breast and non-small cell lung cancer with high-dose epirubicin single agent treatment or high-dose epirubicin containing regimens [157]. The use of hematopoietic growth factors might further exploit this dose-response relationship [158]. However, it has to be taken into account that mucositis is found to replace granulocytopenia as the early dose-limiting toxicity while cardiotoxicity remains the late dose-limiting factor.

Examples of new anthracyclines meant to overcome resistance are idarubicin, detorubicin and esorubicin [159]. Clinical results of these agents however were moderate; continued search delivered the MA that showed nearly full retention of cytotoxic activity against MDR cell lines. In vitro studies have shown that derivates with 9-alkyl substitutions in the A ring of the chromophore and substitutions at the 3'- and/or 4'-position of the daunosamine unit can overcome resistance against anthracyclines [160, 161]. Examples are the MA which retain equal cytotoxic potency in doxorubicin sensitive and resistant cell lines. In vitro studies showed a 3- to 15-fold increased potency in sensitive cell lines compared to their parent compound, with a further 10-fold increase in potency in vivo as a consequence of activation by microsomal enzymes [20]. The MA possess a morpholino ring incorporating the amino nitrogen of the daunosamine unit at the 3'-position. It is supposed that the charged amine at the 3'-position of the daunosamine sugar interacts with P-glycoprotein. Deaminated

anthracyclines have been found to be more cytotoxic in resistant cell lines than their parent compounds [36]. The MA share their morpholino ring as well as a lack of cross-resistance in doxorubicin resistant P-gp positive and negative cell lines [162].

Since the MAs were found to be far more toxic than their parent compounds and therefore are administered at far lower dosages, it is hoped that these compounds are not cardiotoxic. Much research has been directed towards means of reversing the first elucidated resistance mechanism namely P-glycoprotein. Drug resistance could be overcome when the enhanced efflux of MDR-type drugs is blocked, thus restoring intracellular drug concentrations. Reversal agents, or so-called modulators, are competitive inhibitors of the P-glycoprotein pump. The use of P-glycoprotein antagonists has been extensively studied since Tsuruos' description of successful modification of resistance against vincristine and vinblastine by the calcium blocking agent verapamil [163]. After this report many other compounds have proven their activity as P-glycoprotein antagonists [164]. Primarily calcium antagonists and calmodulin inhibitors were studied. Verapamil has been found to be an effective modulator in hematologic malignancies, but in dosages needed for reversal of MDR (2-6 μmol) the drug can have serious cardiovascular toxicity [165-167]. Verapamil analogs (tiapamil, SDB-ethylenediamine) couple a slightly favorable toxicity profile to a reduced modulating capacity. In clinical studies none of the calcium antagonists D-verapamil, bepidril and nifedipine, selected for their reduced cardiovascular toxicity, appeared to be devoid of cardiovascular side-effects [168-170]. Cyclosporins can also sensitize MDR cells but the drug is not only well known for its immunosuppressive potency, but is also notorious for its toxicity, especially acute and chronic nephrotoxicity.

Although cyclosporin plasma levels required for MDR modulation in vitro could be reached in vivo with acceptable toxicity [171-173], its nonimmunosuppressive analog PSC 833 is now subject of intensive research [174-176]. For amiodarone resistance modifying concentrations can be reached without its known cardiac, pulmonary or hepatic toxicity observed after long-term use. First results of clinical studies report no important clinical toxicity at peak serum levels that can effectively enhance cytotoxicity in vitro [177]. A diverse group of other agents, most of them amphipathic and lipophilic, such as quinidine, dipyridamole and ceftriaxone, were reported to exhibit only limited modifying potency [178-180]. A way to obtain potentially reversing concentrations without serious toxicity could be to combine different reversing agents. In vitro addition and synergism of various combinations of modifying agents has been observed [181,182] but until now no in vivo studies with combinations of modifying agents have been performed. As P-glycoprotein is naturally present in organs involved in excretion (liver, kidneys) an increased AUC of the cytotoxic drug is expected. For verapamil changes in the plasma pharmacokinetic parameters of chemotherapeutic agents were observed with higher peak concentrations, a longer terminal half-life and lower plasma clearance of doxorubicin when administered together with verapamil [183]. This is in contrast to the studies of Gigante et al. who found unchanged pharmacokinetics for doxorubicin if combined with verapamil [184]. Mross et al. described similar pharmacokinetics but a difference in metabolism of epirubicin with higher levels of the 7-deoxy-aglycones when epirubicin is combined with verapamil [185].

In solid tumor patients, a higher AUC for doxorubicin and doxorubicinol was observed in phase I studies of doxorubicin combined with PSC 833 [166, 167]. Early results of in vivo studies of doxorubicin combined with PSC 833, administered orally or iv, confirmed these observations. Toxicity of doxorubicin was shown to be increased; its dose had to be reduced in these combination studies. Increased bone marrow toxicity is supposed to be due to an increase in systemic exposure of the cytostatic drug; modulation of P-glycoprotein present in normal bone marrow does not seem to play an important role [186, 187]. In studies of doxorubicin with a modulator, doxorubicinol plasma levels were increased to levels higher than those of doxorubicin, (up to a 350 % increase in the AUC of doxorubicinol). A selective block of the doxorubicinol metabolism has been supposed because the AUC's of doxorubicin in these studies were found to be less increased. As cytochrome P-450 and NADPH glycosidase are enzymes required for the major metabolic pathway of doxorubicinol it was suggested that these enzymes could be selectively inhibited by resistance modulators [186]. The alcohol metabolite of doxorubicin is the most cardiotoxic of the three anthracycline derivates daunorubicin, doxorubicin and epirubicin and their alcohol metabolites [108, 188]; therefore careful monitoring of cardiac function in these types of studies is indicated. Up to now there are no studies that can distinguish between pharmacokinetic and pharmacodynamic effects of the modulators and differentiate between responses obtained by an increased AUC and by truly modulated resistance [187]. There is a need for studies that can analyze the in vivo intratumor kinetic behaviour of anthracyclines and modulating agents. In vivo visualization of the tumor to observe the influence of modulators on tumor pharmacokinetics will give additional insight. In an animal model an organotechnetium complex (^{99}m-Technetium Sestamibi)with a cellular pharmacokinetic profile comparable to the pharmacokinetic profile of cytotoxic agents allowed the visualization of the influence of modulators on drug uptake and efflux [189]. Currently studies are ongoing with ^{99}m-Technetium Sestamibi, for example in our center in combination with PSC 833. Franssen et al. [190] were able to evaluate pharmacokinetics of daunorubicin in rats using C-11 labeled daunorubicin. The C-11 label allows positron emission tomography (PET) scanning and thus visualization of the tumor kinetics of the anthracycline. Studies in animals bearing P-glycoprotein or MDR positive or negative tumors may enable the evaluation of the effect of resistance modulating agents on the efflux of anthracyclines. For clinical research of modifying agents it is very important that, whenever possible, the P-glycoprotein and MRP status of every patient is recorded before, during and after treatment, and that both chemotherapeutic and modulator pharmacokinetics (ensuring blood concentrations capable of reversing P-glycoprotein are actually achieved) are studied [121]. Moreover, the criteria for calling a tumor P-glycoprotein or MDR positive should be defined before starting further studies with reversal agents. Controlled studies, also in previously untreated patients, should be performed to detect differences in response rates between chemotherapeutic treatments with and without modulators in patients with P-glycoprotein positive tumors [167]. This way the clinical value of a modulator can be clearly determined. McLeod stresses the need of the comparison of equipotent dosages of cytostatic drugs with and without a modulator. This necessitates the prior definition of the MTD of a cytostatic drug with a certain dose of a modulator.

First studies with modulating agents usually had a phase I character entering patients with advanced tumors unlikely to respond, and often failed to screen tumors for P-glycoprotein thus evaluating P-glycoprotein modifying capacity of a drug also in patients with tumors not overexpressing P-glycoprotein. Other studies evaluated responses of equal amounts or a fixed reduction of the dose of cytostatic drugs without determining actually achieved serum concentrations of the cytostatics. These factors could have contributed to the first disappointing results of clinical use of response modifying agents outside the field of

GSH
GS-
LED
MA
MDI
MM
MRI
NAI
PET
RES
RNA

hematological malignancies. Recently, the first large randomized studies meant to modulate P-glycoprotein have been published. One of these is a phase III trial of epirubicin and quinidine in advanced breast cancer that found no difference in toxicity profile, response rate and survival in 223 patients (with unknown P-glycoprotein status) [191]. Other possible ways of P-glycoprotein reversal should also be brought into the search for effective modification of resistance, such as the use of monoclonal antibodies directed against P-glycoprotein [192] and inhibition of mdr1 gene expression by the use of antisense oligonucleotides [193]. As resistance in tumors is usually made up of different resistance mechanisms, full reversal of drug resistance is less likely to be achieved by combining chemotherapy with a drug attacking only one mechanism of resistance. A study performed by Herzog et al. [194] demonstrating increased mdr1 expression within 8 hours after treatment of human colon carcinoma cells with the calcium channel blockers verapamil, nifedipine, nicardipine, diltiazem, and cyclosporin A, stresses the urge for a combined approach of tumor cell resistance. Up to now no clinically applicable modulator of MRP is known.

Conclusions

Anthracyclines have gained a major place in curative and palliative chemotherapeutic cancer treatment with activity in a wide scope of neoplasms. As toxicity and primary or secondary resistance limits the value of anthracyclines as anticancer agents, extensive research has been directed towards means of reducing toxicity and increasing sensitivity. Cardioprotective drugs are tested for their ability to reduce the incidence and severity of the chronic toxicity of anthracyclines. High-dose treatments of the less toxic second-generation epirubicin with or without the aid of bone marrow stimulating agents to maximize response rates are ongoing. Drug carriers are tested for more specific delivery and hence more specific cytotoxicity. Current studies are evaluating third-generation derivates with supposed improved toxicity profile and retained activity in resistant tumors. Meanwhile, search for new derivates continues. Growing understanding of the structure-activity relationship will allow more rationally chosen sites and types of derivation. A main goal for the future will be the inclusion of tumors up to now known as primary resistant to chemotherapeutic agents with high incidence, such as non-small cell lung cancer and colon carcinoma, in the therapeutic spectrum of the anthracyclines.

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List Of Abbrevations

ATP	Adenosine triphosphate
AUC	Area under the curve
CHF	Congestive heart failure
DNA	Deoxyribonucleic acid
GSH	Glutathione
GST	Glutathione S-transferase
LED	Liposome encapsulated doxorubicin
MA	Morpholinyl anthracyclines
MDR	Multidrug resistance
MMD	Methoxymorpholinyl anthracyclines
MRP	Multidrug resistance associated protein
NADPH	Nicotinamide-adenine dinucleotide phosphate
PET	Positron emission tomography
RES	Reticuloendothelial system
RNA	Ribonucleic acid

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This is to acknowledge that the slide shown on the front cover of this journal was kindly supplied by Prof. Luciano Majol, University "Federico II", Naples, Italy. It shows the wireframe molecular model of the 2:1 complex of MEN 10706 with the dodecamer $d(CGCAAATTTGCG)_2$, as determined by a combination of 2D-NMR spectroscopy and restrained molecular mechanics and dynamics.